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# Co-Development of *Encarsia formosa* (Hymenoptera: Aphelinidae) and the Greenhouse Whitefly, *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae): A Histological Examination

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Using histological techniques, we have simultaneously examined the co-development of the Aphelinid parasitoid *Encarsia formosa* and its host the greenhouse whitefly, *Trialeurodes vaporariorum*. Previously we have determined that regardless of the whitefly instar parasitized, parasitoid larvae would not molt to their final instar until the whitefly reaches its maximum dimensions. In unparasitized *T. vaporariorum*, this point in development corresponds to the initiation of the adult molt. In part, this study was conducted to determine the developmental state of parasitized whiteflies at the time they achieve their maximum dimensions. It was found that parasitized final instar *T. vaporariorum* do, in fact, undergo a final molt and that *E. formosa* larvae will not molt to their final instar until this has occurred. The timing of the final whitefly molt appears unaffected by parasitization. The commonly observed melanization of parasitized whiteflies appears to be a consequence of this molt. In addition, we have discovered that the adult wasp oviposits within the ventral ganglion of the whitefly, and that major organ systems of the whitefly persist very late into parasitoid development. We also report the presence of possible endosymbiotic bacteria residing in the fatbody of *E. formosa*. Arch. Insect Biochem. Physiol. 51:13–26, 2002. Published 2002 Wiley-Liss, Inc.†

## INTRODUCTION

*Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) has long been a focal point for research in the biological control of whiteflies, and has been used successfully in controlling the greenhouse whitefly, *Trialeurodes vaporariorum* Westwood in greenhouses. Although there has been substantial research on the use of *E. formosa* in biological control strategies, little research has been done on the basic biology and physiology of these insects.

Parasitoids commonly manipulate the physiology and development of their hosts in order to maximize their own success and survival. These strategies include both the injection of viruses, venoms, or regulatory factors at the time of oviposition (Lawrence and Lanzrein, 1993; Lavine and Beckage, 1995; Strand and Pech, 1995; Beckage, 1997) or the release of regulatory compounds by the developing parasitoid (Brown et al., 1993; Schepers et al., 1998; Gelman et al., 1998).

Nechols and Tauber (1977a) demonstrated that the host instar in which parasitization occurred influenced the rate of development of *E. formosa* in *T. vaporariorum*. Parasitization of earlier whitefly instars resulted in longer developmental times for the parasitoids. In addition, they determined that parasitization arrested whitefly development in the

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"transitional" substage of the 4th instar, which would seem to correspond to the mid 4th instar through nymphal-adult apolysis. We have recently studied the effect of host age on the development of *E. formosa*, and also found that the developmental period of parasitoid larvae increased when earlier instars of *T. vaporariorum* were parasitized (Hu et al., 2002). Interestingly, it appeared that the parasitoid larvae alter their own rate of development so that they never reach their final instar until the host has reached its maximum size, which corresponds to the time of the adult molt in unparasitized insects. This study placed parasitoid development within the context of a developmental staging system for whitefly nymphs based on body thickness (Gelman et al., 2002). However, we recognized that the presence of a developing parasitoid within the whitefly might well affect the body thickness of the whitefly, and lead to erroneous conclusions regarding the developmental state of the host. Here we present a complimentary histological study that demonstrates that whiteflies parasitized as early 3rd instars molt at the end of the 4th instar, that the timing of this molt is apparently unaffected by the presence of the parasitoid, and that the parasitoid does not molt to its final instar until after the whitefly has initiated this final molt. Adult development of the whitefly was initiated in some cases.

In addition to determining the relative timing of parasitoid and host developmental events, we were interested in the placement of the egg within the host, the behavioral habits of the parasitoid within its host (i.e., parasitoid feeding, location, and orientation), and the state of the whitefly throughout the development of the parasitoid.

## MATERIALS AND METHODS

*T. vaporariorum* and *E. formosa* were cultured as described in our previous report (Hu et al., 2002). Green bean leaf cuttings infested with *T. vaporariorum* were exposed to *E. formosa* when the whiteflies had reached the early 3rd instar. The instar was identified by measuring body length and width, and young 3rd instars were identified by their flat appearance (Nechols and Tauber, 1977b; Gelman et al., 2002). Oviposition by *E. formosa* was observed under a stereo microscope, and parasitized *T. vaporariorum* were marked by placing a small dot next to each with a fine-tip Gel-Writer pen (Paper-Mate, Japan). Multiple parasitizations of a given nymph were discouraged. Parasitized whiteflies were then kept in an incubator at 26 ± 2°C and a photoperiod of 16:8 L D.

Parasitized whiteflies were collected on a daily basis following oviposition and fixed in Carnoy's no. 2; 60% ethanol:30% chloroform:10% glacial acetic acid (Davenport, 1960) for 2–3 h. The fixed nymphs were washed with absolute ethanol, stained with 1% eosin b in absolute ethanol for 30 min, and washed again with absolute ethanol to remove free eosin; this step stains the nymphs pink, allowing them to be more easily manipulated during embedding. The dehydrated nymphs were then transferred through 4 changes of xylene and placed in paraffin (Paraplast Xtra) at 60°C overnight. After transfer to fresh paraffin in embedding molds, the whiteflies were oriented and chilled rapidly in ice water.

The embedded nymphs were sectioned at 5 μm on a rotary microtome. Sections were relaxed on water at 40°C, mounted on albumin-coated slides, dried, and placed horizontally in a drying oven at 40°C overnight.

Mounted sections were deparaffinized in 3 changes of xylene, transferred through three changes of absolute ethanol, and rehydrated through a series of aqueous ethanol solutions (95, 90, 70, and

Fig. 1. Sections of *T. vaporariorum* ventral ganglion containing *E. formosa* eggs. **A:** Horizontal section of a *T. vaporariorum* ventral ganglion containing a day 0 *E. formosa* embryo. **B:** Sagittal section of a *T. vaporariorum* ventral ganglion containing a day 1 *E. formosa* embryo. **C:** Horizontal section of a ventral ganglion containing a day 0 *E. formosa* embryo and a non-viable egg. **D:** Sagittal section of a non-viable egg within the cortex of a pharate adult whitefly ventral ganglion on day 8 post-oviposition (note normal adult flight muscle development). e, embryo; vg, ventral ganglion; fm, flight muscle; asterisks, non-viable eggs. Scale bars = 50 μm for A–D.

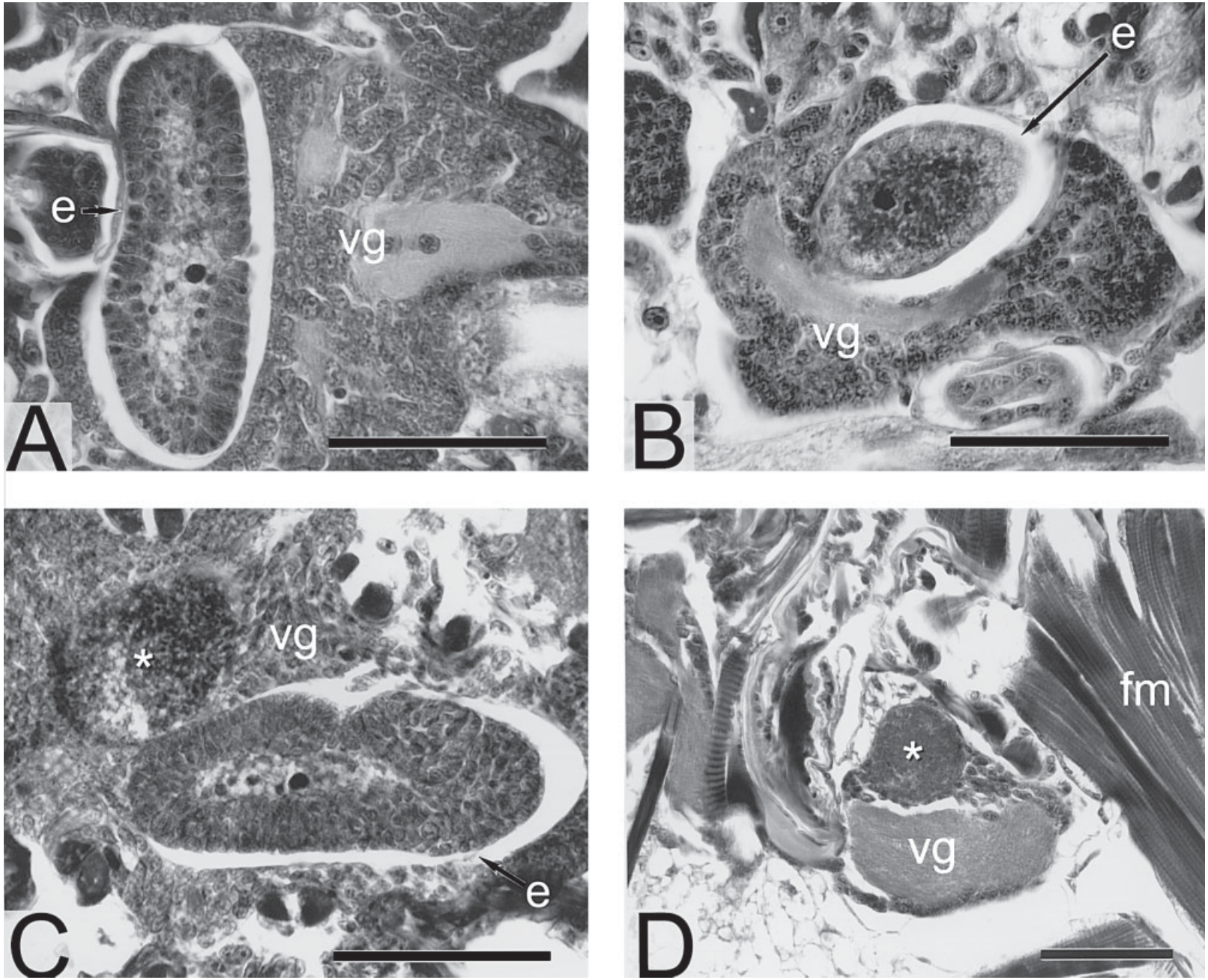


Figure 1.

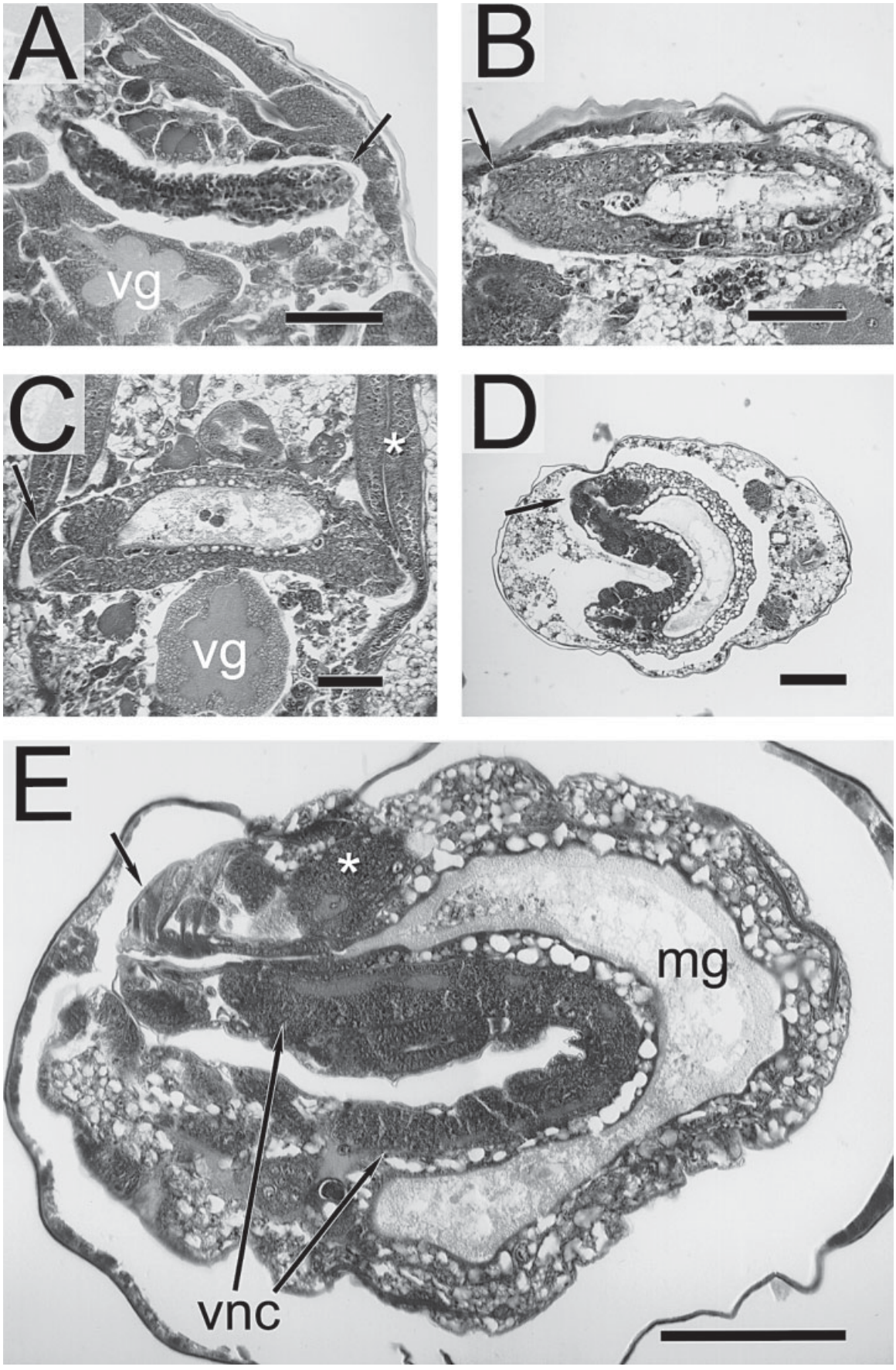


Figure 2.

50%). Sections were stained with Weigert's iron hematoxylin followed by Casson's trichrome as described by Kiernan (1990).

Sections were examined with a Nikon Eclipse 600 compound microscope equipped with Differential Interference Contrast optics. Photomicrographs were taken with a Nikon DMX 1200 CCD camera. Snodgrass (1935) was employed in the identification of internal anatomical structures.

## RESULTS

### Days 0–3

*E. formosa* nearly always deposit their eggs within the dorsal surface of the whitefly ventral ganglion (the fused thoracic and abdominal ganglia); among the 14 embryos observed in this study, only one was not associated with the ventral ganglion. Sections typically showed that embryos were largely surrounded by nervous tissue (Fig. 1A—C). Dimensions of newly deposited embryos were 90–115 μm × 35–40 μm (n = 10). Many whiteflies contained two eggs, however, one of these was always considerably smaller and failed to develop (Fig. 1C). Multiple parasitoid larvae were never observed. First instar larvae began to appear on day 3, post-oviposition. The 1st instar larvae (Fig. 2A) were highly mobile and were observed at locations throughout the body of the whitefly. Early first instar larvae were approximately 30–40 μm (n = 5) in cross section when measured just anterior to the beginning of the midgut. Whiteflies exhibited no observable abnormalities following oviposition or emergence of 1st instar parasitoids; whiteflies were observed molting to the 4th instar by day 3 post-oviposition.

Fig. 2. Developmental stages of *E. formosa* within *T. vaporariorum*. **A:** Early first instar larvae on day 3. **B:** Late 1st instar larvae on day 5. **C:** Early 2nd instar larvae on day 5. Asterisk marks whitefly wingbud. **D:** Late 2nd instar larvae on day 5. **E:** Late 3rd instar larvae on day 9. Asterisk, larval brain; mg, midgut; vnc, ventral nerve cord. Arrows indicate the head of the parasitoid larvae. Scale bars = 50 μm for A–C, 100 μm for D,E.

### Days 4–5

Days 4 and 5 post-oviposition were characterized by a mixture of 1st and 2nd instar parasitoid larvae. It is difficult to distinguish late 1st instar (Fig. 2B) from early 2nd instar larvae (Fig. 2C); however, 1st instar larvae possess hook-like mouthparts that are often somewhat exposed, while 2nd instar larvae possess less prominent mouthparts, a more distinct nervous system, and a midgut that extends farther forward in the body. It is helpful to have longitudinal sections for differentiating a late 1st instar from an early 2nd, but this rarely occurs as larvae at these stages are found in all orientations within the whitefly. Cross-sectional widths of parasitoid larvae measured at the junction of the foregut and midgut were 30–65 μm (n = 5) on day 4, and 55–75 μm (n = 5) on day 5 (one day 5 individual measured 150 μm). There was a greater tendency for the parasitoids to be located more centrally within the whiteflies on days 4 and 5.

The first abnormalities in whitefly tissues were observed on days 4 and 5; the fatbody appeared denser with the appearance of many small cells, and globular masses of cells began to appear in the thoracic region of the whitefly nymphs. It appears that these cellular masses arise from developing flight muscle that has become detached from apodemes; some of these masses displayed faint striations.

### Days 6–7

This period was characterized by a number of rather dramatic changes in both the parasitoid and host. Both 2nd and 3rd instar parasitoid larvae (Fig. 2D and E, respectively) were observed on days 6–7. While all day 6 parasitoid larvae observed were in the second instar (n = 6), 6 of 7 day 7 parasitoid larvae observed were in the 3rd instar. Third instar larvae were easily distinguished from 2nd instar larvae by examination of the head region. Pharyngeal dilator muscles that appeared during the 2nd instar became thicker in the 3rd instar, the pharynx was shifted anteriorly, and the

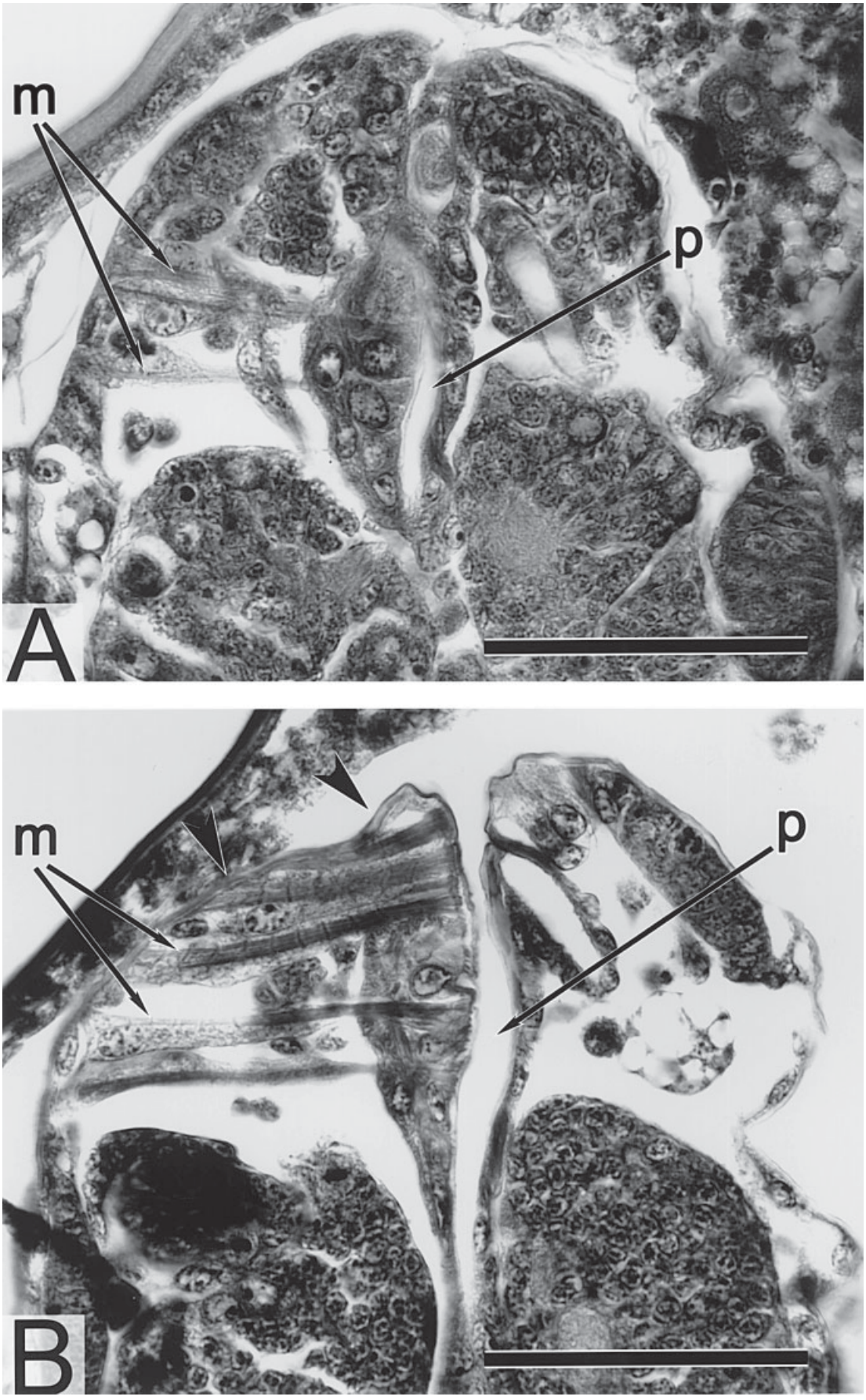


Figure 3.

cuticle of the cephalic region (particularly around the oral cavity) became much thicker (Fig. 3). Growth of the parasitoid larvae was explosive during the early 2nd instar (between day 5 and day 6 post parasitization); day 6 parasitoid larvae ranged in size from 120–160 µm (n = 6) at the junction of the foregut and midgut, while day 7 larvae were found to be 120–210 µm (n = 7). On days 6 and 7, 2nd instar larvae were found to be 120–160 µm (n = 7), while 3rd instar larvae were found to be 150–210 µm (n = 6). The larger parasitoid larvae observed on days 6–7 had taken positions in the central dorsum of the whitefly. Parasitoids were found to "lie on their sides" within the whitefly, that is, the sagittal plane of a parasitoid larva was identical to the horizontal plane of the whitefly (see Fig. 2E). The internal space of the whitefly becomes a limiting factor for parasitoid orientation, and the parasitoid larvae are forced to bend along their ventral surface. Late 2nd instar parasitoids were found to be comma-shaped, while 3rd instar larvae were typically sharply curled into a U-shape and oriented with their head and posterior pointing anteriorly within the whitefly. Second and 3rd instar larvae had extensive fatbody and a prominent midgut. Typically, the contents of the midgut appeared to be a homogeneous material that survives the entire histological procedure. Although many parasitoid larvae had some solid material in their midguts, this material was typically observed in the anterior midgut and may represent material ingested during fixation. It appears that the parasitoids do not feed directly on whitefly tissues extensively at this stage of development. Curiously, we commonly observed what appeared to be rod-shaped bacilli in the fatbody of 3rd instar parasitoids that stained bright red with the acid fuchsin component of Casson's trichrome (Fig. 4). These bacteria, which were approximately 5–8 µm in length, were found throughout the body, but were often particularly abundant in fatbody immediately posterior to the brain.

During days 6–7, whitefly wingbuds were typically absent or actively disintegrating. On day 6, where only 2nd instar parasitoid larvae were observed, one third of the whiteflies had intact wingbuds, one third had no wingbuds, and in one third the wingbuds were actively disintegrating (n = 6). In those cases where the wingbuds were actively disintegrating, rounded epithelial cells were observed dispersing into the whitefly body cavity (Fig. 5C). Although less obvious, disintegration of the developing whitefly eye was also observed (Fig. 5D). On day 7, where most of the parasitoid larvae observed were in the 3rd instar (6 of 7 specimens), whitefly wingbuds were entirely absent. In no case was a 3rd instar parasitoid observed in a whitefly with wingbuds. In some cases, a double layer of cuticle could be detected in whiteflies without wingbuds, indicating that a molt had occurred. (Fig. 6A). The internal organs of the whitefly were generally intact, but with the parasitoid occupying an ever-greater space in the dorsum, organs were compressed ventrally. It is during this period (particularly day 7) that the whiteflies often begin to melanize.

## Days 8–10

Nearly all parasitoids were found to be in the 3rd instar by day 8; these larvae were 150–220 µm (n = 10) in diameter. Larval orientations were similar to that observed on days 6–7. Parasitoids began to pupate on day 8 (2 of 13 specimens), and the majority had pupated by day 10 (5 of 8). The orientation of parasitoid larvae is maintained through the larval-pupal molt (Fig. 7A). However, at pupal eclosion, the parasitoid rotates 90° so that the pupa is facing the ventrum of the whitefly and heads of the parasitoid pupae were found to point anteriorly within the whitefly (Fig. 7B).

Most whiteflies had melanized by day 8. Melanized whiteflies were found to float when placed in the fixative; examination of darkened whiteflies under a stereomicroscope revealed what appeared

Fig. 3. Sagittal sections of 2nd instar (**A**) and 3rd instar (**B**) *E. formosa.* m, pharyngeal dilator muscles; p, pharynx. Arrowheads in B mark the heavier cuticle of the 3rd instar head. Scale bars = 50 µm.

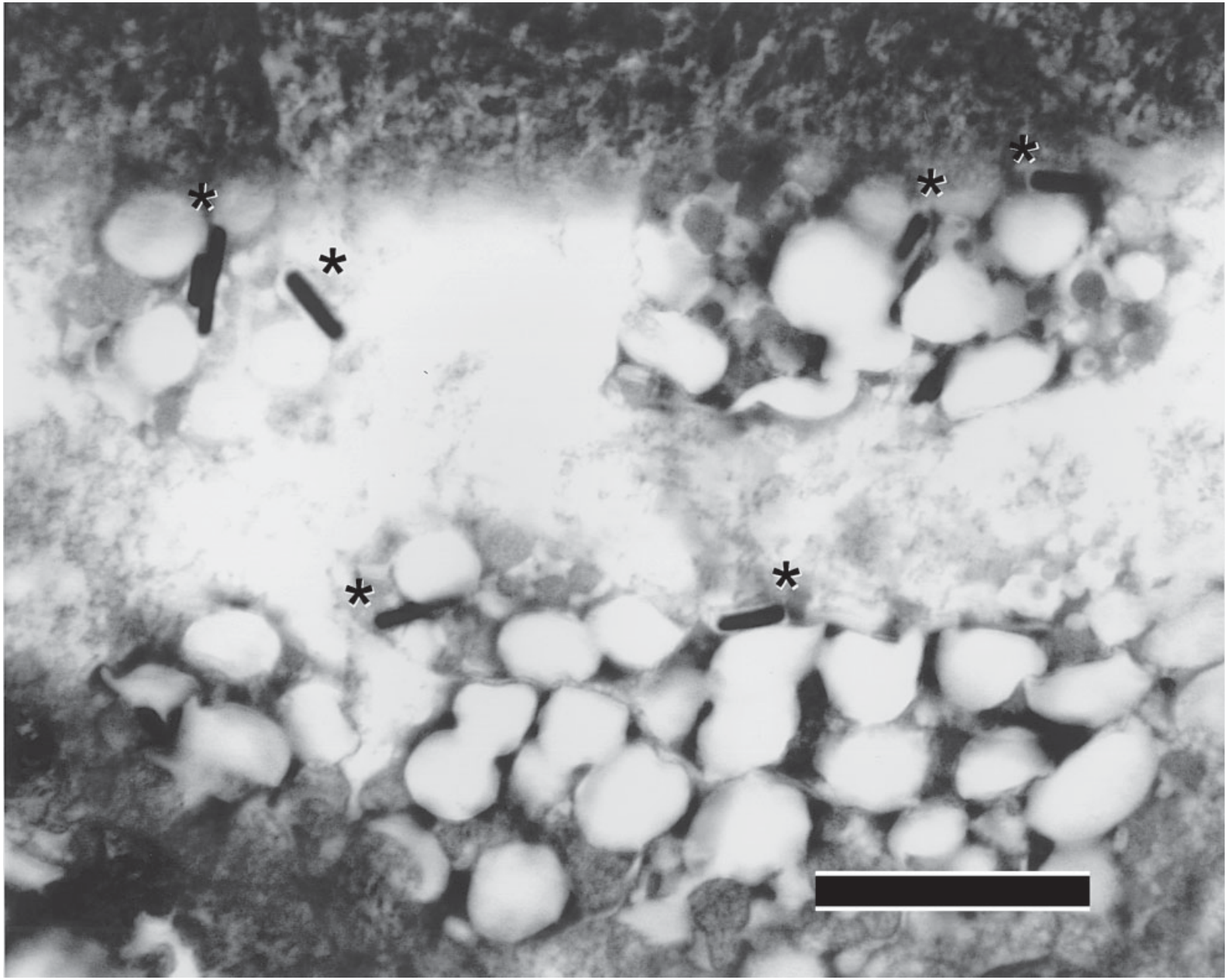

Fig. 4. Apparent bacilli commonly observed in the fatbody of 3rd instar *E. formosa* (asterisks). Scale bar = 20 µm.

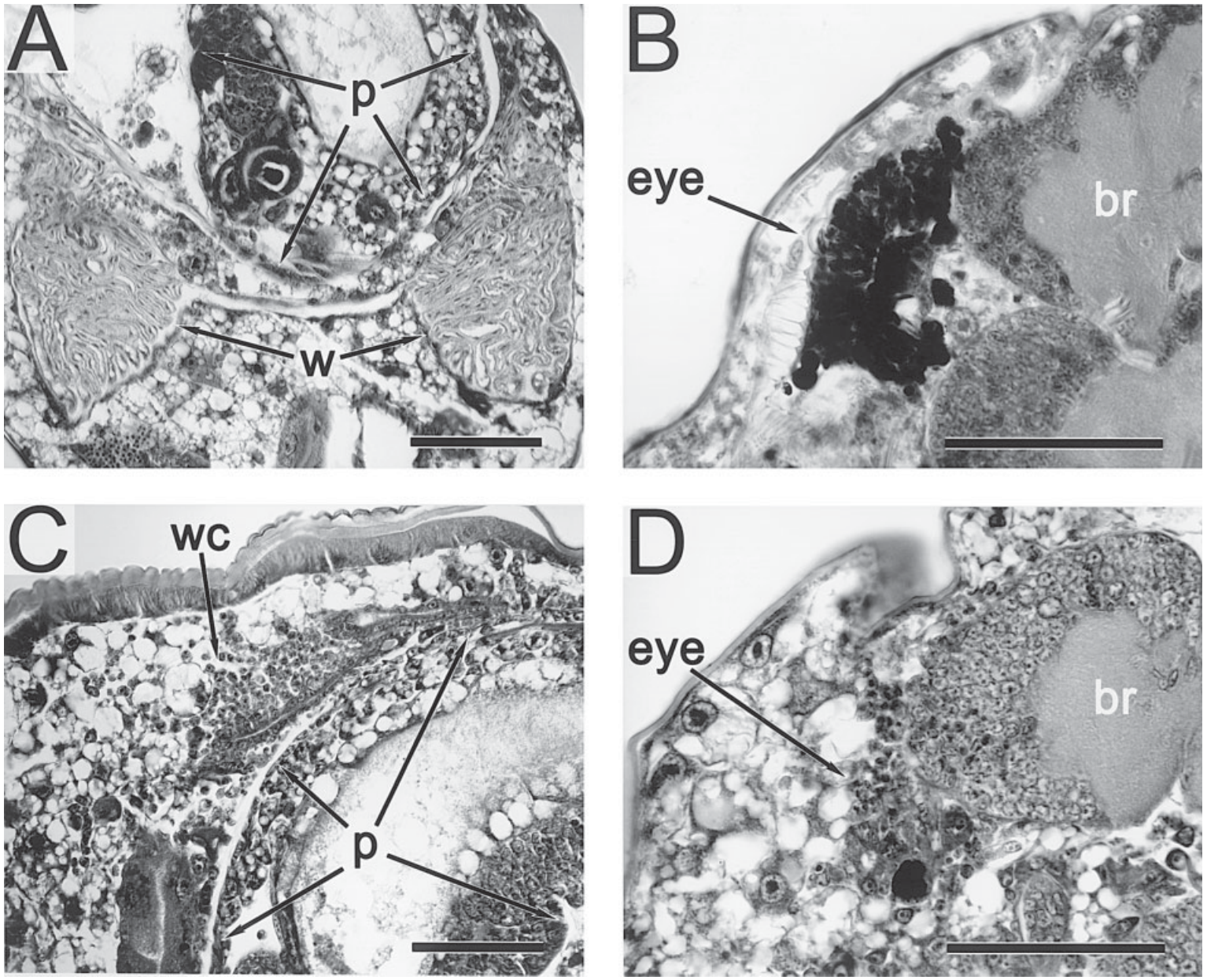


Fig. 5. Fate of wing and eye structures in parasitized *T. vaporariorum*. **A:** Wings of a day 8 parasitized whitefly showing the convoluted folding characteristic of adult development. w, wings; p, parasitoid tissue. **B:** Eye of a day 8 parasitized whitefly showing adult development (br, whitefly brain). **C:** Disintegrating wingbud of a whitefly containing a 2nd instar parasitoid on day 6. wc, mass of detached wing cells; p, parasitoid tissue. **D:** Malformed eye of a whitefly containing a 2nd instar parasitoid on day 6. br, whitefly brain. Scale bars = 50 µm.

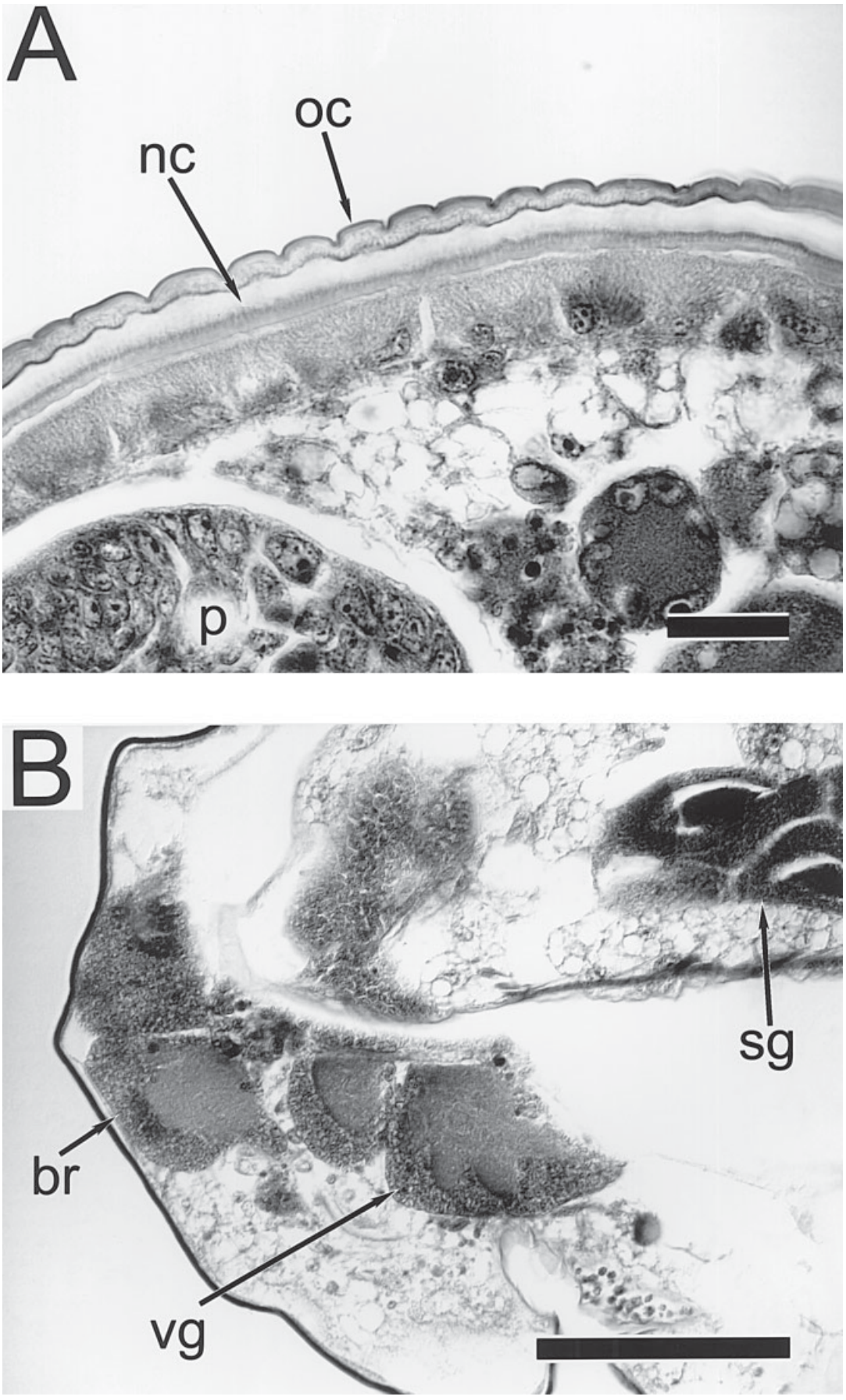


Figure 6.

to be air trapped under the 4th instar cuticle. During the embedding process, the 4th instar cuticle peeled away, revealing a well-formed but apparently immature whitefly. Melanization appeared to be beneath the new cuticle, in the epidermal layer. Melanized whiteflies revealed an absence of wing-buds and eye structures. The fatbody appeared dense, and contained many small cells that probably arose from the disintegration of the wing buds. During the late 3rd instar of the parasitoid (days 8–9), remaining whitefly tissues clearly began to disappear, with fatbody disappearing first. There was a greater tendency for the parasitoid larvae to have solid material within the midgut, although the gut contents were still largely homogeneous in appearance. Most tissues had been cleared from the whitefly prior to pupation of the parasitoid, but it was not unusual to observe late 3rd instar parasitoids, and even pupae in hosts with some internal organs remaining (Fig. 6B). The whitefly nervous system, gut, and mycetomes were conserved until very late in parasitoid development. In several day 8 preparations, the whitefly had clearly initiated adult development, i.e., adult wing and eye development were observed (Fig. 5A and B). These particular preparations contained 3rd instar parasitoid larvae.

## DISCUSSION

*E. formosa* oviposits within the ventral ganglion of the whitefly. The significance of the oviposition site in terms of parasitoid success is not known. However, it is possible that by largely surrounding the egg in host nervous system, an encapsulation response is inhibited. It is also possible that the parasitoid embryo manipulates or receives developmental cues from the host nervous system. Undeveloped eggs were commonly observed in the ventral ganglion; they are easily distinguished from viable embryos by their small size and relatively dense appearance. Because multiple oviposition was discouraged in this study, it appears that the occurrence of multiple eggs within the host is due to a single visit by a wasp. The fact that one egg is invariably small and apparently non-viable further suggests that the wasp may be capable of distinguishing between the deposition of a non-viable and a normal egg. The occurrence of apparently normal pharate adult whiteflies containing infertile eggs suggests that no factors affecting host development are injected at the time of oviposition (Fig. 1D). It is possible that emergence of adult whiteflies might be affected, since no adult whiteflies were examined for evidence of oviposition.

Essential organ systems of the whitefly, such as the nervous system, gut, and mycetomes are often preserved late into parasitoid development, while expendable tissues such as the fatbody, epidermal structures, and gonads are greatly reduced or absent. Thus, it appears that *E. formosa* generally keeps its host "alive" until just prior to pupation of the wasp (or longer). It may be that the parasitoid either remains dependent on nutrients that the host is obtaining from the plant, or that necrosis of whitefly tissues might adversely affect the developing wasp. It is not clear whether the disappearance of "nonessential" whitefly tissues such as the fatbody is due to direct feeding by the parasitoid, or atrophy of these tissues in supplying nutrition to both the parasite and host. Based on the contents of the parasitoid midgut, there is little evidence that parasitoids feed directly on host tissue; the midgut is generally filled with a homogeneous material that solidifies on fixation. There is seldom any appreciable quantity of particulate material in the parasitoid midgut. However, the lining of the oral cavity and pharynx of 3rd instar parasitoids is chitinous, and the mouth and pharynx well muscled, which may allow the larvae to rapidly homogenize ingested tissues.

Gelman et al. (2002) developed a precise stag-

Fig. 6. **A:** A day 6 parasitized whitefly demonstrating the development of a new cuticular layer beneath the 4th instar cuticle. nc, new cuticle; oc, old cuticle; p, parasite tissue. **B:** The nervous system of a day 9 whitefly containing a 3rd instar parasitoid. The head of the parasite is pressed against the nervous system. br, whitefly brain; vg, whitefly ventral ganglion; sg, salivary gland of the parasite. Scale bars = 20 µm (A) and 100 µm (B).

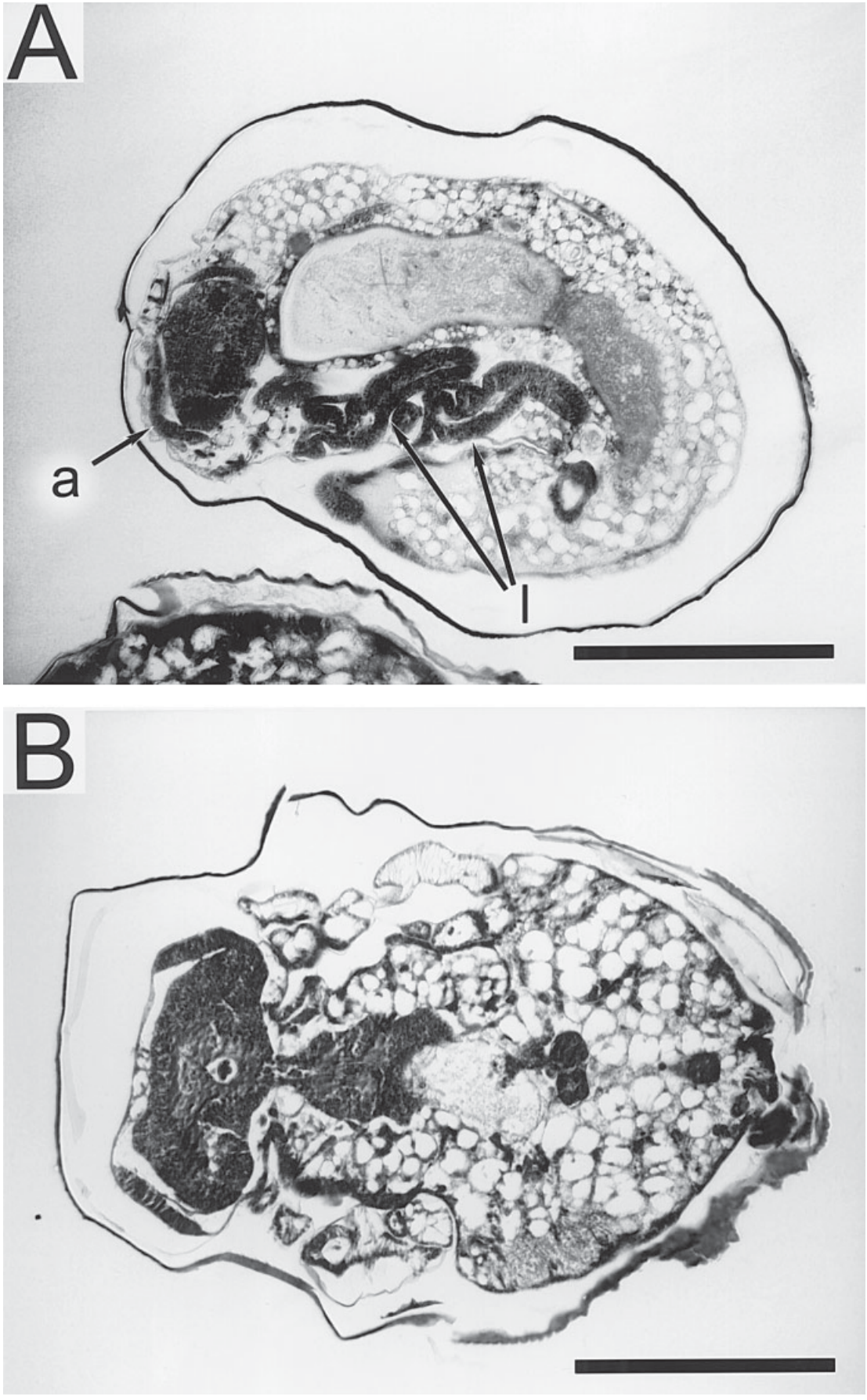


Figure 7.

ing system for identifying developmentally synchronous 4th instar *T. vaporariorum*. In this system, a Stage-5 nymph has reached its maximum body depth, exhibits peak whole body ecdysteroid levels encountered during the 4th instar, and is initiating the nymphal-adult molt. Utilizing this staging system, Hu et al. (2002) reported that regardless of which *T. vaporariorum* instar is parasitized, *E. formosa* larvae will not molt to the 3rd instar until the whitefly has reached Stage 5 of the 4th instar. This seems to indicate that the parasitoid larvae are cueing on either the body size of the host, the host endocrine state, or both. However, we realized that a Stage 5 parasitized nymph might not correspond developmentally to a Stage 5 unparasitized nymph, because the staging criterion is based solely on body depth. Here we report that parasitized 4th instar *T. vaporariorum* undergo a final molt before succumbing to the parasitoid. In some instances, this molt leads to adult characteristics, such as eye and wing development. In most cases, however, the final molt is defective and leads to a whitefly that appears externally to be immature. Internally, a number of epidermal structures that normally differentiate to produce adult cuticular structures, such as the eyes and wingbuds, disintegrate into individual cells that scatter throughout the body. In either case, the whitefly remains encased within the 4th instar cuticle. We found that both 2nd and 3rd instar *E. formosa* could be found in *T. vaporariorum* with disintegrated wingbuds or with adult wing formation. However, we never found a 3rd instar parasitoid in a whitefly *with* intact, immature, wingbuds. Thus, it appears that *E. formosa* is cueing on host endocrine signals in order to correctly synchronize its final development with that of its host.

The developmental rates of parasitized and unparasitized *T. vaporariorum* nymphs seem identical. Gelman et al. (2002) reported that the duration of the *T. vaporariorum* 3rd instar was 3 days, and the duration of the 4th instar to Stage 5 was 4–5 days. Thus, a total of 7–8 days was required for the combined 3rd and 4th instars. In this study, under identical environmental conditions, *T. vaporariorum* parasitized as early 3rd instar nymphs lost their wing structures and began to darken on days 6–7 post-parasitization, indicating that the timing of the whitefly molt was unchanged. Nechols and Tauber (1977b) reported similar findings with regard to the earlier stages of *T. vaporariorum* development: The development rates of 2nd, 3rd, and early 4th instar *T. vaporariorum* parasitized by *E. formosa* did not differ from unparasitized whiteflies. When these immature stages of *T. vaporariorum* were parasitized, host development was arrested in the "transitional" substage of the 4th instar, which includes the initiation of the adult molt (Gelman et al., 2002). Nechols and Tauber (1977a) also reported that melanization of parasitized *T. vaporariorum* coincided with pupation of *E. formosa* and signaled the death of the whitefly. In this study, we found that *T. vaporariorum* parasitized as early 3rd instars complete the 4th instar and undergo a final molt that corresponds temporally to the normal nymphal-adult molt. This final molt is generally defective, resulting in disintegration of differentiating epidermal structures, but can occasionally lead to adult characteristics. Melanization of the whitefly is probably a consequence of the defective molting process. In recently melanized whiteflies, we found primarily 3rd instar parasitoids, and whitefly organs that appeared viable.

Since our colony of *E. formosa* did not exhibit any obvious signs of disease, the bacilli observed in the fatbody of parasitoid larvae may represent a novel endosymbiont. Based on their size and morphology, these bacteria are not the newly described "*Encarsia* bacterium," which has been found in the ovaries of parthenogenetic strains of *Encarsia* (Zchori-Fein et al., 2001).

In summary, the absence of 3rd instar parasitoids in all 4th instar whitefly stages prior to Stage 5, when host ecdysteroid titers are highest and the adult molt has begun, suggests that the parasitoid's molt to the last instar is in some way dependent on the metamorphic molt of the whitefly. The lar-

Fig. 7. **A:** A day 10 parasitoid undergoing pupal development. a, antenna; l, developing leg structures. **B:** A day 9 parasitoid pupa. Scale bars = 200 µm for A,B.

vae of *E. formosa* are capable of such explosive growth that they do not need to manipulate the developmental rate of their host unless late 4th instar or pharate adult whiteflies are parasitized (Nechols and Tauber, 1977b). Rather, the parasitoids time their own development such that their final larval instar develops within the host stage that provides the maximum possible space and nutrient availability. The consistency of the oviposition site, i.e., the ventral ganglion, suggests that the parasitoid egg is in some way dependent upon the host ventral ganglion, although the nature of this dependence is not clear.

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